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(54) Title: DECONTAMINATION OF WHOLE BLOOD AND CELLULAR COMPONENTS BY PHENTHIAZIN-5-IUM-DYES PLUS LIGHT (57) Abstract <p>This invention provides a method for decontaminating blood and cellular blood components by treating the blood, blood component, or compositions containing the blood or blood component with a phenthiazin-5-ium dye and light for a sufficient time to inactivate any pathogenic contaminants. The method of this invention inactivates pathogenic contaminants without substantially altering the blood or cellular blood components such that they are suitable for transfusion.</p>		

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TITLE OF THE INVENTION

DECONTAMINATION OF WHOLE BLOOD AND CELLULAR
COMPONENTS BY PHENTHIAZIN-5-IUM-DYES PLUS LIGHTFIELD OF INVENTION

5 This invention is directed to methods for
inactivating viruses and other pathogenic contaminants
in transfusable blood and blood components.

BACKGROUND OF INVENTION

10 Among the risks to both transfusion recipients
and personnel which are inherent in handling,
transfusing, or receiving blood, blood proteins, or
other blood components is the risk of infection from
pathogenic contaminants, including human
15 immunodeficiency viruses (HIV) and hepatitis viruses.
Virucidal methods, including heat, solvent-detergent,
and gamma irradiation have been used to produce non-
infectious plasma derivatives, but such methods are
either ineffective or too harsh to be used for the
decontamination of whole blood, red cells or
20 platelets. Any treatment that damages or introduces
harmful or undesirable contaminants into the whole
blood or blood components is unsuitable to
decontaminate a product intended for transfusion.

25 Because of the critical need for transfusable red
blood cells and platelets, it is of great importance
to develop methods that can be readily used to
decontaminate cellular blood components and whole
blood without substantially or irreversibly altering
or harming them.

30 To be acceptable for transfusion, at least 75% of
the red cells must be circulating 24 hours after the

-2-

transfusion. The shelf-life and suitability of red blood cells for transfusion is determined on this basis. The concentrations of ATP and 2,3 diphosphoglycerate (2,3 DPG) and the morphology of red cells serve as indicators of the suitability of such cells for transfusion. During prolonged storage and/or as a result of harsh treatments, human red blood cells undergo changes that include decreases in the cellular levels of ATP and 2,3 DPG and changes in cellular morphology. For example, during storage, the concentration of ATP, after a brief initial rise, progressively declines to about 50% of its initial level. The fluidity of the cell membranes of red cells, which is essential for their passage through the narrow channels in the spleen and liver, correlates with their levels of ATP. As the level of ATP declines, the fluidity of the cellular membrane decreases rendering the cells unsuitable for transfusion. The level of 2,3 DPG falls rapidly after about 3 or 4 days of storage and approaches zero after about 10 days. 2,3 DPG is associated with the ability of the hemoglobin in the red cells to deliver oxygen to the tissues.

Solutions that prolong the shelf life of red cells are known (see e.g., Meryman et al., U.S. Patent No. 4,585,735, incorporated herein by reference). Typically such solutions contain citrate, phosphate, glucose, adenine and other ingredients and function to prolong shelf life by maintaining the levels of ATP and 2,3 DPG in the cells. Solutions that contain a penetrating salt, such as ammonium acetate, in addition to phosphate, glucose, and adenine and that are hypotonic with respect to molecules that are unable to penetrate the cell membrane, have been shown

-3-

to maintain the levels of ATP for more than 100 days of refrigeration (see, Meryman et al., supra.).

5 Decontamination treatments that inactivate pathogens, but that do not harm the cellular fractions of blood are not readily available. Presently used decontamination procedures include photosensitizers, which, in the presence of oxygen and upon exposure to wavelengths of light absorbed by the photosensitizer, inactivate viruses (see, e.g., EP 0 196 515, published 10 08.10.86., to Baxter Travenol Laboratories, Inc.). Such compounds include psoralen derivatives (see, e.g., U.S. Patent No. 4,748,120 to Wieseahn), porphyrin derivatives (see, e.g., U.S. Patent No. 4,878,891 to Judy et al.) and other photosensitizers. 15 Often, however, such treatment damages cellular blood components.

The virucidal activity of these compounds is realized when the absorption spectrum of the photosensitizer does not significantly overlap the 20 absorption spectrum of pigments present in the blood. In order to minimize cellular damage, it is advantageous if the photosensitizer is not toxic to red cells and platelets and selectively binds to a component of the virus that is either not present in 25 the red cells or platelets or, if present therein, that is not essential to the red cells' or platelets' function. It is also preferable if the photodynamic treatment inactivates extracellular and intracellular virus as well as cells containing provirus. It is 30 beneficial if the virucidal activity of the photosensitizer is not inhibited by the presence of plasma proteins.

-4-

Photosensitizers such as the psoralens (see, U.S. Patent No. 4,748,120 to Wieseahn) damage nucleic acids in the presence of light while the porphyrins (see, e.g., U.S. Patent No. 4,878,891 to Judy et al.) and merocyanine 540 (MC540) (see, e.g., U.S. Patent No. 4,775,625 to Sieber) cause membrane damage in the presence of light and oxygen and thereby inactivate viruses and bacteriophages.

Among the problems that occur during decontamination with photosensitizers is that they bind to blood components, such as albumin (see, e.g., Prodouz, Transfusion 29:42S (1989)). Prodouz studied the effect of MC540 on platelets and the influence of albumin on the virucidal activity of MC540. In the presence of light and MC540 the platelets aggregated. Albumin prevented the platelet aggregation. Albumin also inhibited the inactivation of viral contaminants because MC540 preferentially binds to albumin, thereby inhibiting the virucidal activity of MC540 plus light.

As the plasma concentration increases, the percentage of viral inactivation substantially decreases. Therefore, because of the competitive inhibition between the binding of dye to plasma proteins and viruses, other dyes have not been suitable for decontaminating blood, cellular blood components, or any blood derived products containing high plasma concentrations.

The phenthiazin-5-ium dyes, which include methylene blue, toluidine blue O, thionin, azure A, azure B, and azure C, are useful for inactivating animal viruses (see, e.g., U.S. Patent Nos 4, 407,282, 4,402,318, 4,305,390 and 4,181,128 to Swartz). However, these dyes have not been used to inactivate

-5-

5 pathogens in whole blood or in cellular blood components because red cells readily take up or bind such dyes (see, e.g., Sass et al., J. Lab. Clin. Med. 73:744-752 (1969)). In addition, methylene blue damages guanine residues of nucleic acids (Simon et al., J. Mol. Biol. 4:488-499 (1962)) and also produces 8-hydroxyguanine (Flyod et al., Arch. Biochim. Biophys. 273:106-111 (1989)). In addition, Girotti demonstrated that: 1) photosensitized oxidation of biological membranes is deleterious to membrane structure and function; and 2) methylene blue cross-links the membrane protein, spectrin, in erythrocytes exposed to visible light and oxygen (Biochim. Biophys. Acta 602:45-56 (1980)). Thus, because of these and other potentially deleterious effects, phentiazin-5-ium dyes have not been used as photosensitizers for decontaminating blood or cellular blood components.

20 None of the decontamination methods has proven fully successful for decontaminating whole blood or compositions containing concentrated blood components, including those with high levels of plasma. There is, however, an acute need to develop a safe method whereby pathogenic contaminants, particularly HIV and hepatitis, in blood or in cellular blood components can be inactivated without rendering the blood or cellular blood component unsuitable for transfusion.

SUMMARY OF THE INVENTION

30 It is one object of this invention to provide an improved method for inactivating pathogenic contaminants in transfusable compositions, comprising: adding an effective amount of at least one phentiazin-5-ium dye to a transfusable composition,

-6-

wherein said amount is effective for inactivating substantially all of said pathogenic contaminants; and, treating said composition with an effective amount of light having an effective intensity,
5 duration and wavelength, whereby substantially all of said pathogenic contaminants are inactivated.

It is another object of this invention to provide a method for decontaminating compositions that contain blood or cellular blood components, comprising: adding
10 an effective concentration of at least one phenthiazin-5-ium dye to said composition; and treating said composition for a sufficient length of time with light, which includes an effective wavelength, to inactivate any pathogenic contaminants
15 in said composition, wherein said effective concentration of dye is sufficient to inactivate said pathogenic contaminants is a concentration that is acceptable for transfusion, said sufficient length of time is sufficient to inactivate said components
20 without substantially or irreversibly harming said blood or cellular blood components, and said effective wavelength is preferentially absorbed by said dye, whereby said pathogenic contaminants are inactivated but said blood or blood components are suitable for
25 transfusion.

It is another object of this invention to provide a method for decontaminating blood or cellular blood components, comprising: adding an effective
30 concentration of at least one phenthiazin-5-ium dye to said blood or blood component; and treating said blood or blood component for a sufficient length of time with an effective amount of light, which includes an effective wavelength, to inactivate any pathogenic contaminants in said composition, wherein said

-7-

effective concentration of dye is sufficient to inactivate said pathogenic contaminants and is a concentration that is acceptable for transfusion, said sufficient length of time is sufficient to inactivate
5 said components without substantially or irreversibly harming said blood or cellular blood components, said effective amount of light that includes a sufficient amount of an effective wavelength that is preferentially absorbed by said dye, whereby said
10 pathogenic contaminants are inactivated but said blood or blood components are suitable for transfusion.

It is another object of this invention to provide a method for decontaminating compositions that contain high concentrations of plasma, comprising: adding a
15 effective concentration of at least one phenothiazin-5-ium dye to said composition; and treating it for a sufficient length of time with an effective amount of light, which includes an effective wavelength, to inactivate any pathogenic contaminants in said
20 composition, wherein said effective concentration of dye is sufficient to inactivate said pathogenic contaminants and is a concentration that is acceptable for transfusion, said sufficient length of time is sufficient to inactivate said components without
25 substantially or irreversibly harming said plasma, and said effective amount of light includes a sufficient amount of an effective wavelength that is preferentially absorbed by said dye, whereby said pathogenic contaminants are inactivated but said
30 plasma remains suitable for transfusion.

This invention significantly improves the procedure for decontaminating blood and cellular blood components by providing a method that produces non-infectious blood or blood components that can be

-8-

transfused without the need for diluting or removing the photosensitizing dye.

5 In practicing this invention at least one phenthiazin-5-ium dye said dye, such as methylene blue, toluidine O thionin, azure A, azure B, azure C, and any other phenthiazin-5-ium dye known to those of skill in the art, is added to the blood, blood component, plasma, platelet concentrate or composition that contains blood, a blood component, or platelets. 10 The mixture is then treated with an effective wavelength of light, such as red light, and any pathogenic contaminant, such as a viral pathogen, is inactivated.

BRIEF DESCRIPTION OF THE FIGURES

15 Figure 1 presents the dependence of inactivation of bacteriophage $\phi 6$ in 16% solution of plasma in Unisol as a function of methylene blue concentration in the sample. Samples containing the bacteriophage, plasma and increasing concentrations of methylene blue were exposed for 4 minutes at a fluence rate of 2 20 mW/cm² of sample delivered by General Electric F15T8-R bulbs.

25 Figure 2 presents the dependence of the inactivation of bacteriophage $\phi 6$ by 40 μ g/ml amino-methyl-trimethyl psoralen (AMT) as a function of plasma concentration in Unisol. Samples containing the bacteriophage, AMT and increasing concentrations of plasma were exposed to UVA irradiation (wavelength of 365 nm) at a fluence rate 42 mW/cm² for 90 seconds.

-9-

Figure 3 depicts the dependence of bacteriophage $\phi 6$ inactivation in platelet concentrates as a function of leukocyte concentration. Increasing concentrations of leukocytes were added to 4 ml samples containing
5 three log leukocyte-depleted platelet, at about 2×10^8 platelets per ml, $5 \mu\text{M}$ methylene blue and then exposed for 5 minutes to light at a fluence rate of 2 mW/cm^2 of sample delivered by General Electric F15T8-R bulbs.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

10 Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs. All publications
15 mentioned herein are incorporated by reference thereto.

As used herein, a pathogenic contaminant is a contaminant that, upon transfusion or handling of blood or a component thereof, may cause disease in the recipient or handler thereof. Examples of such
20 pathogens include, but are not limited to, retroviruses, such as HIV, and hepatitis viruses.

As used herein, a blood component is a component that is separated from blood and includes, but is not limited to red blood cells, platelets, blood clotting
25 factors, plasma, enzymes, plasminogen, and immunoglobulins. A cellular blood component is a component of blood, such as a red blood cell, that is a cell. A blood protein is a protein that is normally found in blood. Examples of such proteins are blood
30 factors VII, VIII. Such proteins and components are well-known to those of skill in the art.

-10-

As used herein, a composition containing a cellular blood component or a blood protein is a composition that contains a biologically compatible diluent and a blood component, blood protein, or mixtures thereof. Such compositions may also contain plasma and leukocytes. If such compositions are leukodepleted, the concentration of leukocytes is reduced by a specified amount.

As used herein, a transfusable composition is a composition that can be transfused into the blood stream and that contains blood, at least one cellular blood component, concentrated plasma, or mixtures of blood, cellular blood components, and plasma.

As used herein, decontamination refers to a process whereby pathogens, such as viral contaminants, are rendered non-infectious so that blood or a composition that contains blood, a blood component or blood protein can be transfused or manipulated without harming or infecting anyone exposed thereto.

As used herein, a pathogen includes any replicable agent that infects or occurs in blood or blood components. Such pathogens include any virus, bacterium, or parasite known to those of skill in the art to be found in blood or products derived from blood. Examples of pathogens include but are not limited to: bacteria, such as Streptococcus species, Escherichia species, and Bacillus species; viruses, such as human immunodeficiency viruses, other retroviruses, herpes viruses, paramyxoviruses, cytomegaloviruses, hepatitis viruses, including hepatitis A, hepatitis B, and hepatitis C, pox viruses, and toga viruses; and parasites, such as

-11-

malarial parasites, including Plasmodium species, and trypanosomal parasites.

5 As used herein, the ratio of the titer of the control sample to the titer of virus in each of the treated samples, is herein called virus inactivation. The \log_{10} of this ratio is herein called \log_{10} inactivation. Typically, a \log_{10} of inactivation of at least about 5 to 6 logs indicates that the treated sample has been decontaminated.

10 As used herein, a composition in which substantially all of the contaminating pathogens have been inactivated is one in which the concentration of active pathogen has been decreased by a factor of at least about 5 to 6 logs. A composition in which
15 substantially all of the contaminating pathogens have been inactivated is, thus, decontaminated.

As used herein, fluence is a measure of the energy per unit area of sample and is typically measured in joules/cm². Fluence rate is a measure of
20 the wattage, of light that strikes a unit area of the sample. For example, it can be measured as milliwatts (mW)/ per cm². Fluence rate can also be expressed as the amount of energy that strikes the sample in a given amount of time and may be measured as joules/cm²
25 per unit time of exposure.

As used herein, a phenothiazin-5-ium dye includes any dye that one having skill in the art would consider a member of that class. This class includes, but is not limited to, methylene blue, toluidine blue
30 O, thionin, and azure A, B and C.

-12-

As used herein, plasma can be prepared by any method known to those of skill in the art. For example, it can be prepared by centrifuging blood at a force that pellets the cells and forms an interface between the red cells, the buffy coat, which contains leukocytes, and above which is the plasma. Depending on centrifugation conditions, the number of leukocytes and platelets in the plasma can vary.

As used herein, leukocyte depleted platelets or red cells are components that have been passed through a filter that decreases the concentration of leukocytes by a factor of 10^2 to 10^5 . Such filters are identified by the log of the factor by which the blood component is depleted of leukocytes.

As used herein, extracellular pH is the pH of the medium in which red blood cells or other cellular blood components are stored or maintained.

As used herein, a biologically compatible solution or a biologically compatible buffered solution is a buffered solution in which cells that are contacted therewith retain viability. Contacting includes any process in which the cells are in some manner exposed to the buffered solution and includes, but is not limited to, suspension of the cells in the buffered solution. A biologically compatible buffered solution has a pH and a salt concentration that is suitable for maintaining the integrity of the cell membrane. Such a solution does not inhibit or destroy the biological and physiological reactions of the cells contacted therewith. Typically a biologically compatible buffered solution has a pH between 5 and 8.5 and is isotonic or only moderately hypotonic or hypertonic. Biologically compatible buffered

-13-

solutions are readily available to those of skill in the art. Examples of biologically compatible buffered solutions include, but are not limited to those listed in Table I, infra.

5 As a first step when practicing any of the
embodiments of the invention disclosed herein, blood
is drawn from a donor into an anticoagulant solution,
such as CDPA-1, and may then be washed or prepared for
storage in a suitable, biologically compatible,
10 buffered solution, such as ARC 8 (Meryman et al., Vox
Sang 18:81-98 (1991)), or any that are well-known to
those of skill in the art. The whole blood may then
be subjected to a decontamination process in
accordance with this invention. Alternatively, the
15 blood may be separated into its components, including,
but not limited to, plasma, platelets and red blood
cells, by any method known to those of skill in the
art. For example, blood can be centrifuged for a
sufficient time and at a sufficient centrifugal force
20 to form a pellet containing the red blood cells.
Leukocytes collect primarily at the interface of the
pellet and supernatant in the buffy coat region. The
supernatant, which contains plasma, platelets, and
other blood components, may then be removed and
25 centrifuged at a higher centrifugal force, whereby the
platelets pellet.

 Human blood normally contains about 7×10^9
leukocytes per liter. The concentration of
leukocytes, which pellet with the red cells, can be
30 decreased by filtering through a filter that decreases
their concentration by selected orders of magnitude.
Leukocytes can also be removed from each of the
components by filtration through an appropriate filter
that removes them from the solution. When practicing

-14-

the method of this invention, if leukocytes are not removed from the composition that is being treated, the concentration of dye, the light intensity, and/or the time of irradiation must be somewhat increased.

5 It is well within the level of skill in the art to ascertain the amount by which any or all of such parameters should be adjusted. It has, however, been discovered that plasma proteins do not affect the inactivation reactions that occur when practicing the

10 method of this invention. Thus, the values of these parameters need not be adjusted for the presence of plasma proteins.

In accordance with this invention, the composition of blood, cellular blood components, or concentrated plasma, a composition containing blood,

15 cellular blood components or mixtures of cellular blood components, plasma and leukocytes or any other composition containing blood or blood components, may be obtained or prepared as described above or by any

20 means or method known to those of skill in the art.

In one embodiment of this invention such compositions are obtained in, prepared or introduced into gas permeable blood preservation bags, which are sealed and flattened to a width sufficiently narrow to

25 permit light to pass through and irradiate the contents, whereby any pathogen present in the bag would be irradiated. Any such blood bag known to those of skill in the art may be used as long as there is sufficient oxygen present in the bag to react with

30 the photosensitizer and the bag is transparent to the selected wavelength of light.

The composition that is decontaminated may include any suitable biologically compatible

-15-

physiological solution known to those of skill in the art. Examples of such solutions include, but are not limited to Unisol and ARC 8 (see, TABLE 1, infra.).

5 The dyes or photosensitizer compounds of this invention include the phenothiazin-5-ium dyes. Any such dye known to those of skill in the art may be used. Examples of such dyes include, but are not limited to, methylene blue, toluidine blue O, azure A, azure B, azure C and thionin. An effective amount of
10 at least one selected dye is introduced into the composition. Ideally the selected dye is non-toxic and the effective concentration is acceptable for transfusion so that the treated blood or blood component does not require additional manipulation to
15 remove the dye and thereby risk contamination.

 The effective concentration of dye to be used can be determined by one of skill in the art. Generally it is in the range, but is not limited to, 0.2 to 50 μM .

20 In a preferred embodiment methylene blue may be selected. Methylene blue is used therapeutically to treat methemoglobinemia at a dosage of 1 mg/kg of body weight to a maximum recommended dosage of 2 mg/kg. Thus, blood or cellular blood components or other
25 compositions treated in accordance with this invention can be directly transfused, as long as the final dosage of methylene blue is less than about 2 mg/kg of body weight.

 In a preferred embodiment of this invention
30 methylene blue is introduced into the composition at a concentration of about 1 μM to about 25 μM . Thus, when used in accordance with this invention, the

-16-

amount of methylene blue needed for inactivation is substantially less, about twenty-five fold less, than the maximum recommended dosage. For example, transfusion of ten units of red cells at a 55% hematocrit to a 70 kg individual that have been treated with 5 μ M methylene blue in accordance with this invention would only provide a dose of methylene blue of 0.08 mg/kg, which is substantially less than the maximum recommended dosage.

The mixture of the blood or blood component composition and dye is then irradiated for a sufficient time with an appropriate wavelength or mixture of wavelengths, whereby pathogenic contaminants in the composition are inactivated. Such wavelength is one that is absorbed by the dye, but that does not damage the blood or blood components present in the composition. It is well within the level of skill in the art to select such wavelength and to ascertain a sufficient time for inactivation. For example, the selected wavelength is based on the absorption profile of the selected dye or dyes and is one that does not substantially damage the cellular components of the composition selected for decontamination. Further, model viral systems are known to those of skill in the art. These model systems may be used to test the selected dye and light source. Such model viral systems include, but are not limited to the enveloped bacteriophage, bacteriophage ϕ 6, vesicular stomatitis virus (VSV), which is an animal virus that contains its genome encoded as DNA, and Sindbis virus, which is an animal virus that contains its genome encoded as RNA. Based on the effective values of parameters, such as wavelength and light intensity, measured for such model systems, one having skill in the art can select the values for

-17-

these parameters for use in practice. For example, one having skill in the art would know that if the intensity or power of the light source is decreased, a greater concentration of dye and/or longer exposure times should be used.

In one embodiment of this invention red blood cells, which have been leukodepleted with a five log filter, are suspended in ARC 8 at a hematocrit of about 15 to 55% introduced into gas permeable blood preservation bags in an amount such that the filled bag has a thickness of about 4 mm, and treated with methylene blue at a concentration of about 1 μ M up to about 25 μ M and red light of wavelength (560 to 800 nm.) at a sufficient intensity and for a long enough time, such as for about 60 minutes at 3.6 joule/cm², to inactivate pathogenic contaminants in the red blood cells. The virucidal activity of the methylene blue and light treatment is not affected by the presence of up to 100% plasma but was reduced by the presence of leukocytes. Accordingly, in the presence of leukocytes, light intensity, dye concentration, and/or irradiation time must be increased in order to ensure that the sample is decontaminated.

In other embodiments of this invention compositions containing platelets and compositions containing high concentrations of plasma may be decontaminated by exposure for a sufficient time to an effective concentration of a phenothiazin-5-ium dye plus an effective amount of an appropriate wavelength of light.

Following treatment in accordance with the method of this invention, the blood, cellular blood component or composition may be stored or transfused.

-18-

Alternatively, after treatment of compositions such as red cell preparations or platelet-rich plasma, the composition can be centrifuged at a force sufficient to pellet the cellular components. The supernatant can then be removed following centrifugation and the cells resuspended to reduce the concentration of residual photosensitizer and any reaction products.

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

TABLE 1

TYPICAL BIOLOGICALLY COMPATIBLE ANTICOAGULANT AND
CELL PRESERVATION SOLUTIONS

SOLUTION CONCENTRATION

	INGRED.	CPDA-1 [*] (mM)	UNISOL (mM)	ARC 8 (mM)
15	NaCitrate	89.6	17.3	33.3
	cit. acid	15.6	2.7	-
	glucose	-	-	139
	dextrose	161.0	35.5	-
20	NaH ₂ PO ₄	16.1	-	2.9
	Na ₂ HPO ₄	-	3.0	12.0
	Adenine	2.0	2.2	2.0
	NaCl	-	110.4	-
	KCl	-	5.1	-
25	CaCl ₂	-	1.7	-
	MgCl ₂	-	4.0	-
	NaHCO ₃	-	40.0	-
	pH	5.7	7.4	7.4

^{*}CDPA-1 is sold by Baxter Travenol.

-19-

EXAMPLE 1

Materials:

5 Plasma was prepared from human blood by centrifugation to pellet the red cells and to remove the platelets. Leukocytes were removed by filtration with a log filter as indicated.

Methylene Blue was reagent or USP grade.

Unisol and ARC 8 are prepared as indicated in TABLE I.

10 Blood bags were gas permeable.

Bacteriophage $\phi 6$ stock solution was prepared from lysates of the HB10Y strain of Pseudomonas phaseolicola. At the concentrations used, methylene blue was not harmful to virus in the absence of light.

15 Platelet-poor human plasma was diluted to a final concentration of 16% in Unisol. Forty μ l of bacteriophage $\phi 6$ stock solution was added to 4 ml of sample and varying amounts of 1 mg/ml methylene blue solution were added to each sample. The samples were
20 incubated at room temperature and then exposed for 4 minutes to light delivered by General Electric F15T8-R bulbs at a fluence rate of 2 mW/cm² of sample.

25 After the light treatment, the samples were diluted and the virus was titered by a plaque assay and compared with the number of plaques in a control sample that was not exposed to light. The ratio of the titer of the control sample to the titer of virus in each of the treated samples, is a measure of viral

-20-

inactivation. The \log_{10} of this ratio is herein called \log_{10} inactivation.

5 The results are shown in Figure 1 in which the log of the $\phi 6$ inactivation is plotted versus the negative log of the concentration of methylene blue. The optimal concentration of methylene blue for maximal virus inactivation is about 5 μM .

EXAMPLE 2

10 Forty μl bacteriophage $\phi 6$ and 7.5 μl of 1 mg/ml methylene blue were added to 4 ml samples containing varying concentrations of plasma in Unisol. The concentrations of plasma varied between 2.5% and 100%. The final concentration of methylene blue in each sample was 5 μM . The control sample contained
15 bacteriophage, methylene blue and 100% plasma.

Each sample, except for the control, was exposed to a fluence rate of 2 mW/cm² of the light delivered by General Electric F15T8-R bulbs for 5 minutes. The virus in each sample was titered and the results are
20 set forth in TABLE 2. It can be seen that the method was effective in inactivating virus in plasma at all concentrations, including concentrations of 16% and higher.

25 In contrast to the results set forth in TABLE 2, when viral inactivation using other dyes, including MC540, various psoralens, and various porphyrins, was measured, it was found that inactivation was significantly reduced (up to 10⁵) as a function of increasing plasma concentration (see Figure 2 and
30 TABLE 3).

-21-

In Figure 2, bacteriophage $\phi 6$ was added to increasing amounts of plasma. Forty $\mu\text{g/ml}$ of amino-methyl-trimethyl (AMT) psoralen was added to the plasma-bacteriophage mixture and irradiated with UVA
5 light (wavelength of 365 nm) at a fluence rate of 42 mW/cm^2 for 90 seconds.

TABLE 3 presents the results of experiments in which increasing concentrations of plasma were inoculated with VSV and treated with 6.25 μM MC-540
10 and visible light for 60 minutes. As with bacteriophage $\phi 6$ and AMT (Figure 2), MC-540 is unsuitable for decontaminating blood components that contain high levels of plasma.

As plasma concentration increases, the degree of
15 viral inactivation decreases significantly, which indicated that dyes, other than phenthiazin-5-ium dyes, are unsuitable for decontaminating blood components. Thus, unlike most dyes and photosensitizing compounds, phenthiazin-5-ium dyes are
20 able to decontaminate in the presence of high concentrations of plasma.

Thus, surprisingly, only phenthiazin-5-ium dyes, were able to decontaminate in the presence of high concentrations of plasma. The ineffectiveness of
25 treatment using other dyes is most likely the result of binding of the dye to proteins and other plasma constituents so that at higher plasma concentrations virus binding is competitively inhibited. Surprisingly, however, this does not occur with the
30 phenthiazin-5-ium dyes used in this invention sufficiently to interfere with viral inactivation or, as demonstrated, infra., to substantially harm or alter cellular blood components.

-22-

TABLE 2
PLASMA DEPENDENCE OF INACTIVATION OF BACTERIOPHAGE ϕ 6
BY METHYLENE BLUE

	<u>Plasma</u> <u>Concentration (%)</u>	<u>Titer</u>	<u>log₁₀</u> <u>inactivation</u>
5	100, control	3.4×10^8	---
	2.5	2.6×10^2	6.1
	16	9.9×10^2	5.5
	30	1.2×10^3	5.5
10	50	5.7×10^2	5.8
	100	1.2×10^3	5.5

Control = no light treatment

-23-

TABLE 3

PLASMA DEPENDENCE OF INACTIVATION OF VSV
BY MEROCYANINE 540

	<u>Plasma Concentration</u> <u>(%)</u>	<u>Titer</u>	<u>log₁₀</u> <u>Inactiva-</u> <u>tion</u>
5	<u>Controls:</u>		
	Mock Infection	0	---
	Virus Control	5 x 10 ⁸	---
	Virus in 100% Plasma	2.4 x 10 ⁸	---
10	<u>Virus Treated:</u>		
	0% Plasma	< 8.0 x 10 ¹	>6.4
	6.25% Plasma	< 8.0 x 10 ¹	>6.4
	12.5% Plasma	1.6 x 10 ⁴	4.1
	25% Plasma	4.5 x 10 ⁶	1.8
15	50% Plasma	1.5 x 10 ⁷	1.1
	100% Plasma	1.0 x 10 ⁸	0.1
	Virus: Vesicular Stomatitis Virus		
	Assay: Plaque counts (agarose overlay) on BGMK cells		
20	Treatment: 6.25 μ M MC-540, 60 minutes visible light		

EXAMPLE 3

Red cells were prepared by centrifugation of whole blood to form packed red cells. The supernatant was expressed off. The remaining red cell pellet had a hematocrit of 85-95% (volume percent occupied by red cells).

In this instance, packed red cells were diluted with 0.9% saline to a hematocrit of 55% and then the red cells were leukocyte-depleted by filtration through a three log filter that decreases the leukocyte concentration by a factor of 10³. Aliquots

-24-

of the red cells were diluted with ARC 8 to final hematocrits of 15 or 30% and contained about 2.5% plasma.

5 Forty μ l of bacteriophage ϕ 6 and varying amounts
of 1 mg/ml methylene blue were added to 4 ml samples
of the leukocyte-depleted red cells, which were at a
hematocrit of 15 or 30%). Methylene blue was added to
each sample, except for a control, at final
10 concentrations of .1 μ M, 5 μ M, or 25 μ M. The samples
were then exposed to light, as described in Example 2
for different lengths of time and the titer of the
virus was assayed. The results of this experiment are
summarized in TABLE 4.

15 The experiment was also performed with a red
blood cell sample at a hematocrit of 55% with 5 μ M
methylene blue and 60 minutes of light exposure at a
fluence rate of 0.8 mW/cm². The sample, however, was
placed on a reciprocating shaker during exposure to
the light. The log₁₀ inactivation was 5.7.

-25-

TABLE 4

INACTIVATION OF BACTERIOPHAGE $\phi 6$ IN RED CELLS

	<u>Sample</u>	<u>Titer</u>	<u>log₁₀</u> <u>Inactiva-</u> <u>tion</u>
	<u>Hematocrit 15%:</u>		
5	Control, no MB no light	3.1×10^8	---
	Control, 25 μM MB, no light	3.4×10^8	0.0
10	Control, no MB 16 m. light	2.3×10^8	0.1
	25 μM + 5 m. lt.	10	7.5
	5 μM + 5 m.lt.	5.9×10^2	5.7
	5 μM + 15 m. lt.	< 10	> 7.5
15	1 μM + 5 m.lt.	4.7×10^7	0.8
	1 μM + 15 m.lt.	4.2×10^2	5.9
	1 μM + 25 m.lt.	3.0×10^1	7.2
	<u>Hematocrit 30%:</u>		
	5 μM + 5 m.lt.	2.0×10^8	0.2
20	5 μM + 15 m.lt.	1.1×10^8	0.5
	5 μM + 25 m.lt.	3.6×10^4	3.9
	<u>Hematocrit 55%:</u>		
	5 μM + 30 m.lt. + shaking	6×10^2	5.7

-26-

TABLE 5

INACTIVATION OF VSV IN 16% PLASMA BY METHYLENE BLUE

<u>Sample</u>	<u>Titer</u>	<u>log₁₀</u> <u>inactiva-</u> <u>tion</u>
Control:		
no dye	1.5×10^8	---
no light	2.3×10^7	0.8
15 s. lt.	4.2×10^5	2.6
30 s. lt.	1.4×10^4	4.0
60 s. lt.	$< 8 \times 10^1$	> 6.3

25 μ M Methylene BlueFluence rate: 2 mW/cm² delivered by General
Electric F15T8-R fluorescent
bulbsEXAMPLE 4

The inactivation of VSV in 4 ml samples of 16% plasma/Unisol by 25 μ M methylene blue as a function of time of light exposure was measured. The results are set forth in TABLE 5. VSV is rapidly inactivated in plasma by light plus methylene blue.

EXAMPLE 5

The inactivation of bacteriophage $\phi 6$ in platelet concentrates (PC) and in leukodepleted platelet concentrates (LDPC) was studied. Platelet concentrates contain about 2×10^8 platelets per ml. The concentrates are depleted of leukocytes by filtration as described in Example 3 to produce LDPC.

Forty μ l of bacteriophage $\phi 6$ was added to 4 ml samples of either PC or LDPC, which contained 5 μ M

-27-

methylene blue. The control sample was not exposed to light and the other samples were either exposed to light for 5 minutes or 25 minutes. The results are set forth in TABLE 6.

5 The data in TABLE 6 indicate that viral inactivation is somewhat slower in the presence of leukocytes than in their absence. The presence of leukocytes appears to interfere with the inactivation of viral contaminants in PC so that longer light exposure or higher concentrations of dye were needed to achieve inactivation. The reason for this interference was not clear.

15 In order to ascertain whether methylene blue binds to or is otherwise taken up by leukocytes, leukocytes from the buffy coat were incubated in the presence of methylene blue. After incubation the leukocytes were spun down and the concentration of methylene blue in the supernatant was compared to the concentration of methylene blue in the absence of leukocytes. There was no difference in concentration of methylene blue, which indicated that, although leukocytes contain DNA, they do not bind or take up methylene blue.

25 In contrast, when a similar experiment was conducted with red blood cells, the concentration of methylene blue in the supernatant was substantially less than the initial concentration of methylene blue before the addition of red blood cells. This indicated that red blood cells take up or bind methylene blue so that the experiment with leukocytes should have detected any dye uptake or binding by leukocytes.

-28-

EXAMPLE 6

The inactivation of bacteriophage $\phi 6$ in 16% plasma by 5 μM toluidine blue O, another phenothiazine dye, as function of exposure to light was examined. The results, which are set forth in TABLE 7, indicated that toluidine blue is as effective as methylene blue for the inactivation of bacteriophage $\phi 6$ in plasma.

TABLE 6

INACTIVATION OF BACTERIOPHAGE $\phi 6$
IN PLATELET CONCENTRATES

<u>Sample</u>	<u>Titer</u>	<u>\log_{10}</u> <u>Inactivation</u>
Control	1.6×10^8	---
5 m. lt. (PC)	1.1×10^5	3.2
5 m. lt. (LDPC)	4.5×10^2	5.6
25 m. lt. (PC)	8.2×10^1	6.3
25 m. lt. (LDPC)	1.1×10^1	7.2

5 μM Methylene BlueFluence rate: 2 mW/cm² delivered by General Electric
F15T8-R fluorescent bulbs

PC: platelet concentrate

LDPC: leukodepleted platelet concentrate

-29-

TABLE 7

INACTIVATION OF BACTERIOPHAGE $\phi 6$
IN 16% PLASMA BY TOLUIDINE BLUE O

<u>Sample</u>	<u>Titer</u>	<u>log₁₀</u> <u>inactivation</u>
Control: no light	1.6×10^8	---
1 min. lt.	4.3×10^3	4.6
2 min. lt.	5.5×10^1	6.5
4 min. lt.	2×10^0	7.9

5 μ M Toluidine Blue O
10 Fluence rate: 2 mW/cm² delivered by General Electric
F15T8-R

EXAMPLE 7

15 Since blood for transfusion is generally
collected and stored in blood bags, the inactivation
of bacteriophage $\phi 6$ by methylene blue in red blood
cells in bags was studied. Sufficient amounts of red
blood cell solution were added to gas permeable bags
to fill them to a width of 4 mm. A parallel
20 experiment was conducted with red blood cells at a
thickness of 4 mm in petri dishes. The results of
these experiments are set forth in TABLE 8.

25 The blood bags were filled with 62 ml of red
blood cells, which had been leukodepleted with a 5 log
filter, diluted to a hematocrit of 30%, and inoculated
with bacteriophage $\phi 6$ as in the previous Examples. In
order to prevent any decrease in the light intensity
over the length of the bags, they were sealed with
hemostats, rather than relying on the ports, which
30 would have blocked light flux. Samples were extracted
with a needle through the side of the bag.

-30-

As indicated in the TABLE 8, treatment with methylene blue and light in the gas permeable bags inactivated the phage.

TABLE 8

5 INACTIVATION OF BACTERIOPHAGE ϕ 6 IN RED BLOOD CELLS IN BLOOD BAGS AND PETRI DISHES BY METHYLENE BLUE

	<u>Sample</u>	<u>Titer</u>	<u>\log_{10} inactivation</u>
	<u>Petri dish:</u>		
	no light	8.9×10^8	---
10	30 min. lt.	1.6×10^3	5.7
	60 min. lt.	2.7×10^2	6.5
	<u>Bag:</u>		
	no light	1.1×10^9	---
	30 min. lt.	3.7×10^6	2.5
15	60 min. lt.	3.5×10^4	4.5
	90 min. lt.	1.8×10^3	5.8
	120 min. lt.	1.8×10^2	5.8

20 5 μ M Methylene blue
Gas permeable blood preservation bags were used.
Fluence rate: 0.8 mW/cm² delivered by General Electric F40T8-R bulbs
Red blood cells leukodepleted (with a 5 log filter) and suspended at a hematocrit of 30% in ARC 8.

EXAMPLE 8

25 The ability of the phenthiazin-5-ium dye, thionin, to inactivate bacteriophage ϕ 6 in 100% plasma in the presence of red light was studied. From the results, which are set forth in TABLE 9, it was concluded that thionin is effective for
30 decontaminating whole blood and blood components.

-31-

TABLE 9

INACTIVATION OF BACTERIOPHAGE ϕ 6 IN 100% PLASMA
BY THIONIN AND RED LIGHT

	<u>Sample</u>	<u>Titer</u>	<u>\log_{10}</u> <u>inactivation</u>
5	no light	4.4×10^8	---
	2 min. lt.	3.9×10^5	3.1
	4 min. lt.	3.8×10^4	4.1
	8 min. lt.	2.5×10^3	5.2
	16 min. lt.	4.1×10^2	6.0
10	5 μ M Thionin Fluence rate: 0.8 mW/cm ²		

EXAMPLE 9

15 The inactivation of VSV in red blood cells by methylene blue and light was examined. Samples of red blood cells which had been leukodepleted with a 5 log filter and suspended at a hematocrit of 30% in ARC 8, were inoculated with VSV and were treated with methylene blue at a concentration of 5 μ M. The samples were treated with red light as set forth in

20 TABLE 10. The results of this experiment indicated that VSV in red blood cells was readily inactivated by methylene blue and light.

-32-

TABLE 10

INACTIVATION OF VSV IN RED BLOOD CELLS
BY METHYLENE BLUE AND RED LIGHT

	<u>Sample</u>	<u>Titer</u>	<u>log₁₀</u> <u>inactivation</u>
5	no light	---	---
	15 min. lt.	1.1 x 10 ⁴	4.1
	30 min. lt.	8 x 10 ¹	1.9
	60 min. lt.	1 x 10 ⁰	6.0
	90 min. lt.	no plaques	≥ 6.1
10	120 min. lt.	no plaques	≥ 6.1
5 μ M Methylene blue			
Fluence rate: 0.8 mW/cm ² delivered by General			
Electric F40T8-R bulbs			
15	Red blood cells leukodepleted (with a 5 log filter)		
	and suspended at a hematocrit of 30% in ARC 8.		

EXAMPLE 10

The effect of treatment with methylene blue and light on red blood cells was studied. The results of this experiment are set forth in TABLE 11.

20 Red blood cells were leukodepleted with a 5 log filter and suspended at a hematocrit of 30% in ARC 8.

-33-

TABLE 11

RED CELL VIABILITY AFTER TREATMENT WITH 5 μ M METHYLENE
BLUE AND 90 MINUTES OF LIGHT

	<u>Sample</u>	<u>% Hem</u>	<u>ATP</u> <u>μM/g</u> <u>Hgb</u>	<u>3-DPG</u> <u>μM/g</u> <u>Hgb</u>	<u>EC</u> <u>pH</u>	<u>Morph</u> <u>Score</u>
5	C - 0	0.24	5.8	12.9	7.10	98.8
	T - 0	0.23	6.1	13.0	7.10	98.0
	C - 7	0.36	7.4	16.5	7.11	81.2
	T - 7	0.42	7.8	13.7	7.08	82.7
	C - 14	0.38	7.9	18.6	7.06	84.3
10	T - 14	0.46	7.4	14.6	7.05	81.9
	C - 23	0.53	7.4	21.1	6.83	-
	T - 23	0.71	6.1	18.0	6.90	-
	C - 28	0.53	7.4	24.4	6.67	79.7"
	T - 28	0.73	6.4	16.2	6.75	79.5"
15	C - 36	0.82	6.0	21.2	6.64	77.2
	T - 36	1.11	5.5	12.1	6.71	78.8
	C - 42	0.96	8.5	25.4	6.57	77.2
	T - 42	1.40	7.1	14.5	6.61	79.6

20 Fluence rate: 0.8 mW/cm² delivered by General Electric F40T8-R bulbs
 Red blood cells leukodepleted (with a 5 log filter) and suspended at a hematocrit of 30% in ARC 8.
 C - n = control red blood cells stored for "n" days.
 T - n = treated red blood cells stored for "n" days.

25 *EC = extracellular
 ** assessed on day 30

A 62 ml sample of cells was introduced into a blood bag as described in Example 7 and the blood bag was stored at 4°C. At the times indicated in TABLE 11,

-34-

5 samples were removed from the bag with a needle and the in vitro properties of the cells were assessed. A second 62 ml sample of cells was introduced into a blood bag and stored at 40°C. This second bag was the control.

10 The in vitro properties that were measured included: extracellular pH; percentage of hemolysis; the concentrations of ATP and 2,3-DPG; and morphology. The in vitro properties of the treated and untreated samples were measured and compared to ascertain the effects, if any, of light and methylene blue on stored red blood cells.

15 The results, which are set forth in TABLE 11, indicate that treatment with methylene blue and light did not have a substantial effect on red cells. Thus, red cells that have been decontaminated with methylene blue and light can be used for transfusion. Further, red cells that have been treated and then stored for extended periods of time remain suitable for transfusion.

20

EXAMPLE 11

25 Platelet concentrates were inoculated with VSV or bacteriophage $\phi 6$ and treated with 1 μM MB and 6.4 joules/cm² of red light. Treatment inactivated at least 6 logs of VSV and the phage. The effects of treatment on platelet morphology and the recovery of the treated platelets from hypotonic stress were examined.

-35-

EXAMPLE 12

5 A platelet concentrate in 16% plasma/Unisol was leukodepleted with a three log filter. Three hundred μ l of differing concentrations of leukocytes were added back to 2.7 ml of platelet concentrate. Bacteriophage stock was added as in the previous Examples. Methylene blue was then added to each sample to a final concentration of 5 μ M. Each sample was irradiated for 5 minutes by light having a fluence rate of 2 mW/cm². The results are shown in Figure 3 from which it can be concluded that their presence inhibits viral inactivation by methylene blue.

15 Since modifications will be apparent to those of skill in the art, it is intended that this invention be limited only by the scope of the appended claims.

-36-

We claim:

1. A method for decontaminating transfusable compositions that contain blood or cellular blood components, comprising:
 - 5 adding a effective concentration of at least one phenthiazin-5-ium dye to said composition; and
 - treating said composition for a sufficient length of time with light, which includes an effective wavelength of sufficient intensity,
 - 10 wherein said effective concentration of dye is acceptable for transfusion, said effective wavelength is preferentially absorbed by said dye, and said effective concentration in conjunction with said light and sufficient time inactivates substantially all
 - 15 pathogenic contaminants in said blood or blood components, without substantially or irreversibly harming said blood or said cellular blood components.
2. The method of claim 1, wherein said dye is selected from the group consisting of methylene blue, toluidine O, azure A, azure B, azure C and thionin.
- 20 3. The method of claim 1, wherein said effective concentration of dye is from about 1 μ M up to and including about 25 μ M.
4. The method of claim 1, wherein said light is red light and includes wavelengths from 560 to 800 nanometers.
- 25 5. The method of claim 1, wherein said blood component is selected from the group consisting of red blood cells, platelets, leukocytes and mixtures of any
- 30 of red blood cells, platelets, leukocytes and plasma.

-37-

6. The method of claim 1, wherein the contaminating pathogen(s) in said blood or blood components is at least one pathogen selected from the group consisting of viruses, parasites, and bacteria.

5 7. A method for decontaminating blood or cellular blood components, comprising:

 adding an effective concentration of at least one phentiazin-5-ium dye to said blood or blood component; and

10 treating said blood or blood components for a sufficient length of time with light, which includes an effective wavelength of sufficient intensity, wherein said effective concentration of dye is acceptable for transfusion, said effective wavelength
15 is preferentially absorbed by said dye, and said effective concentration in conjunction with said light and sufficient time inactivates substantially all pathogenic contaminants in said blood or blood components, without substantially or irreversibly
20 harming said blood or said cellular blood components.

8. The method of claim 7, wherein said dye is selected from the group consisting of methylene blue, toluidine O, azure A, azure B, azure C and thionin.

25 9. The method of claim 7, wherein said effective concentration of dye is from about 1 μ M up to and including about 25 μ M.

30 10. The method of claim 7, wherein said the contaminating pathogen(s) in said blood or blood components is at least one pathogen selected from the group consisting of viruses, parasites, and bacteria.

-38-

11. The method of claim 7, wherein said light is red light and includes wavelengths from 560 to 800 nanometers.

5 12. The method of claim 7, wherein said blood component is selected from the group consisting of red blood cells, platelets, leukocytes and mixtures of any of red blood cells, platelets, leukocytes and plasma.

10 13. A method for decontaminating compositions that contain high concentrations of plasma, comprising: adding a effective concentration of at least one phenthiazin-5-ium dye to said composition; and

15 treating said composition for a sufficient length of time with light, which includes an effective wavelength of sufficient intensity, wherein said effective concentration of dye is acceptable for transfusion, said effective wavelength is preferentially absorbed by said dye, and said effective concentration in conjunction with said light
20 and sufficient time inactivates substantially all pathogenic contaminants in said blood or blood components, without substantially or irreversibly harming or altering said composition.

25 14. The method of claim 13, wherein said dye is selected from the group consisting of methylene blue, toluidine O and thionin.

15. The method of claim 13, wherein said effective concentration of dye is from about 1 μM to and including about 25 μM .

-39-

16. The method of claim 13, wherein said light is red light and includes wavelengths from 560 to 800 nanometers.

5 17. The method of claim 13, wherein the contaminating pathogen(s) in said blood or blood components is at least one pathogen selected from the group consisting of viruses, parasites, and bacteria.

10 18. A method for inactivating pathogenic contaminants in transfusable compositions, comprising:
adding an effective amount of at least one phenthiazin-5-ium dye to said transfusable composition, wherein said amount is effective for inactivating substantially all pathogenic contaminants in said blood or blood components; and
15 treating said composition with an effective amount of light having an effective intensity, duration and wavelength, whereby substantially all pathogenic contaminants in said blood or blood components are inactivated.

20 19. A method for inactivating pathogenic contaminants in transfusable compositions that contain leukocytes, comprising:
leukodepleting said composition;
adding a photosensitizing dye to said
25 leukodepleted composition; and
irradiating said composition with light that includes an effective wavelength, intensity and duration, whereby substantially all of the pathogenic contaminants in said transfusable compositions that
30 contain leukocytes are inactivated.

20. The method of claim 19, wherein said pathogenic contaminant is at least one pathogen

-40-

selected from the group consisting of viruses, bacteria, and parasites.

21. The method of claim 20, wherein said viruses includes viruses selected from the group consisting of
5 retroviruses, herpes viruses, hepatitis viruses, pox viruses, paramyxoviruses, toga viruses and cytomegaloviruses and said bacteria include bacteria selected from the group consisting of Streptococcus
species, Escherichia species, and Bacillus species,
10 and said parasites, are selected from the group consisting of malarial parasites and trypanosomal parasites.

1/3

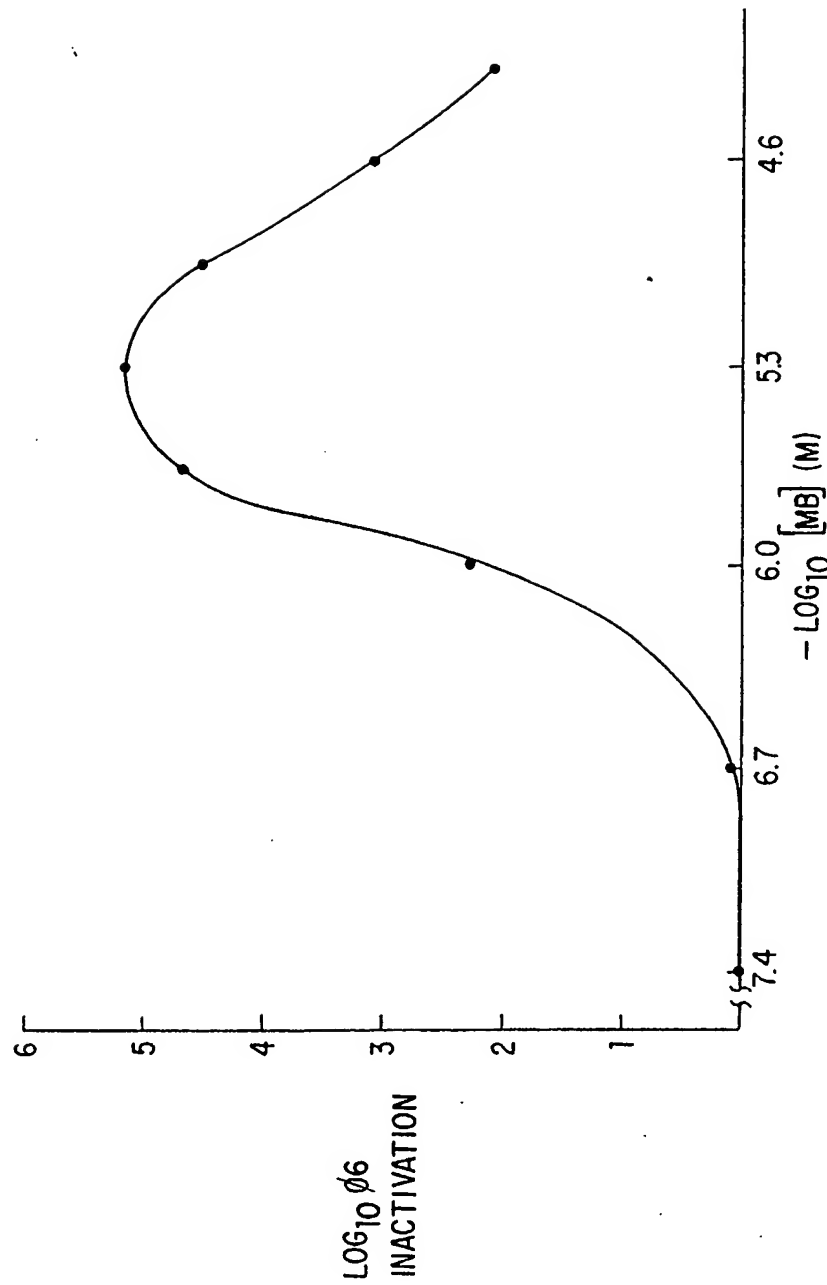


FIG. 1

SUBSTITUTE SHEET

2/3

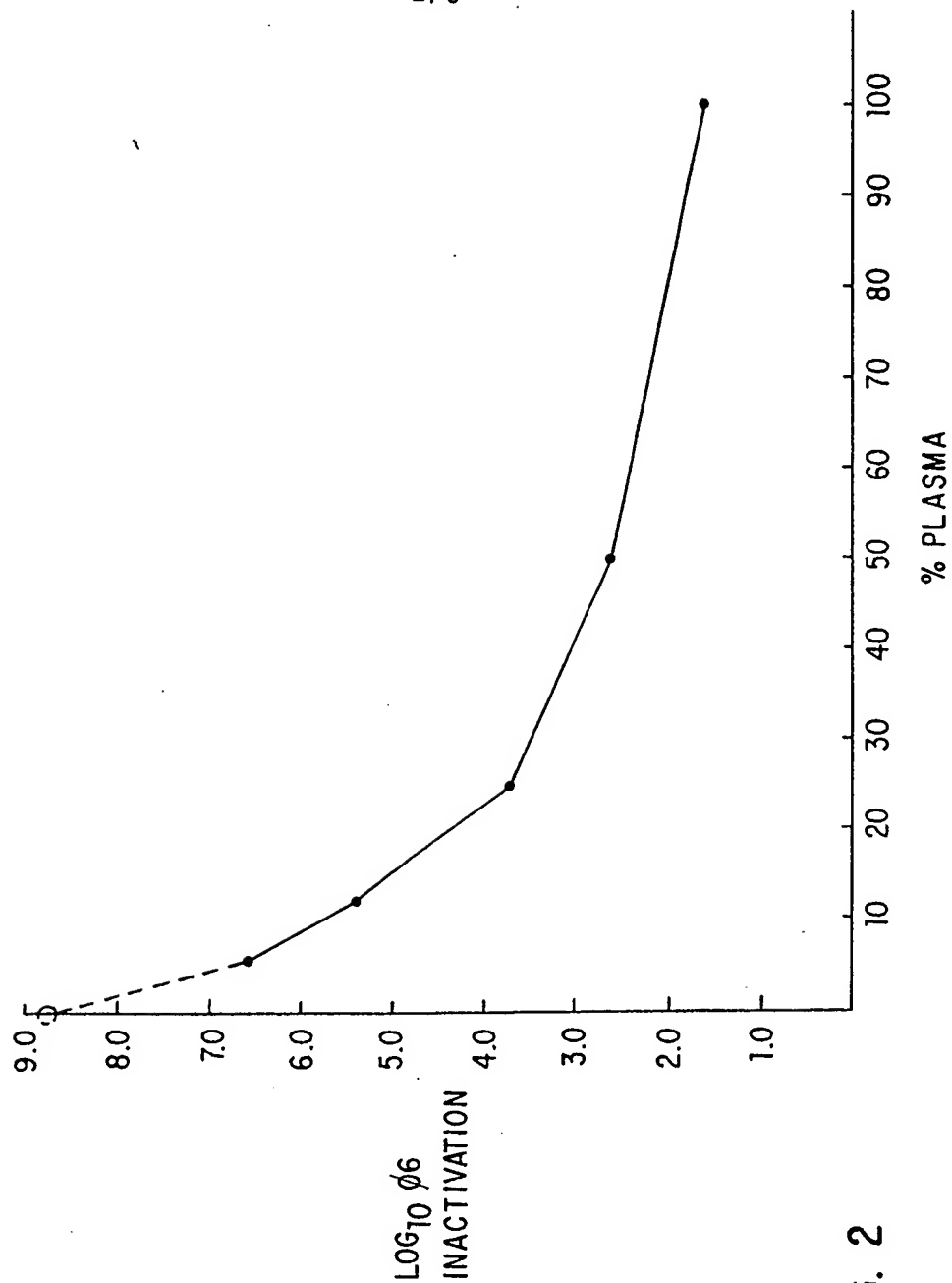


FIG. 2

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3/3

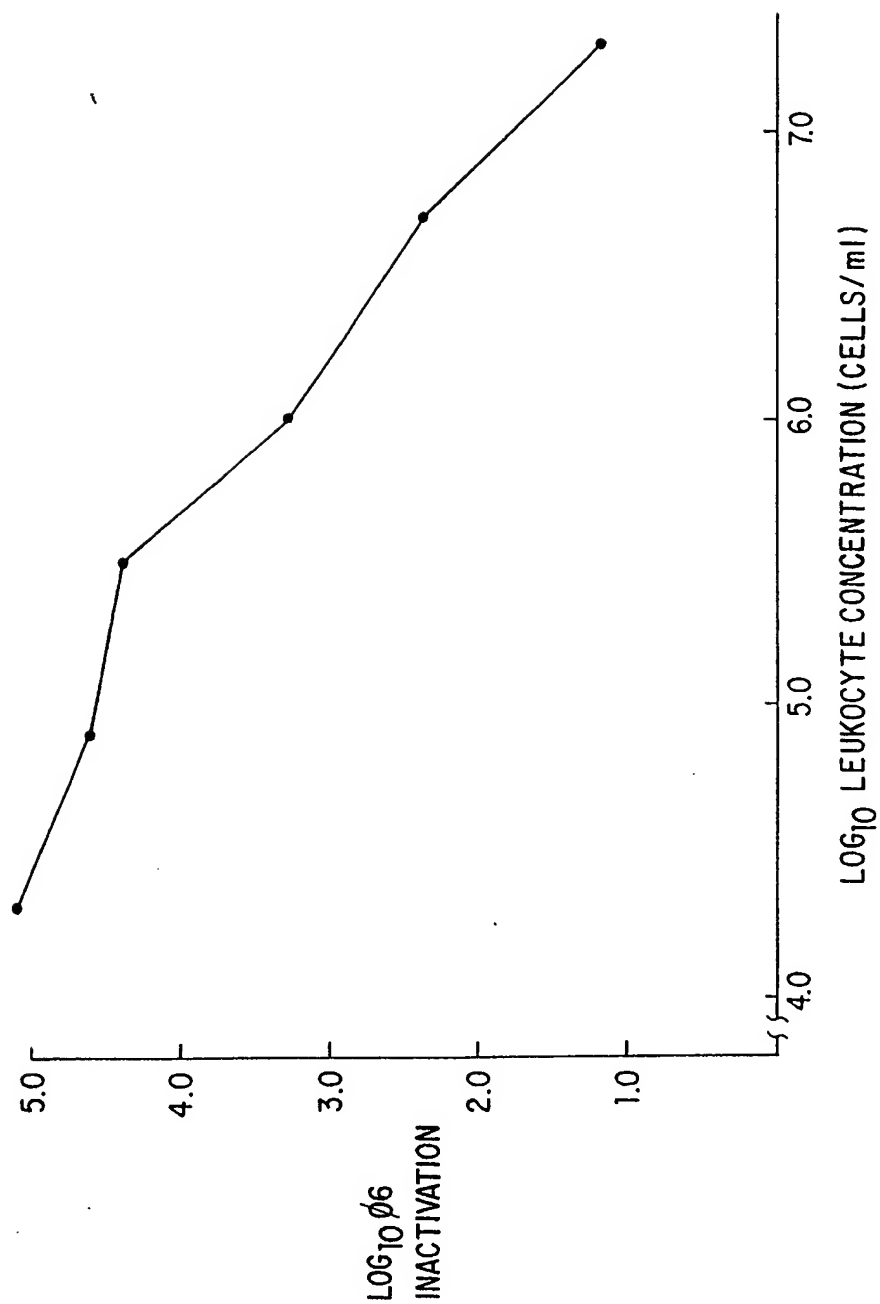


FIG. 3

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/02976

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC(5): A61K 35/14		
U.S. CL.: 435/2, 435/238, 514/224.8		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
U.S.	435/2, 435/238, 514/224.8	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
APS, MEDLINE, WORLD PATS		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	JP, A, 61-275228 (DOLANA) 05 December 1986, see claims 1-20 and page 10 of the English translation.	18-21
Y	US, A, 4,878,891 (JUDY ET AL.) 07 November 1989, see abstract and claims 1-2, 7, 9-16.	1-21
Y	US, A, 4,727,027 (WIESEHAHN ET AL.) 23 February 1988, see abstract.	1-21
Y	US, A, 4,915,683 (SIEBER) 10 April 1990, see abstract.	1-21
Y,P	US, A, 4,950,665 (FLOYD) 21 August 1990, see abstract.	1-21
Y	US, A, 4,305,390 (SWARTZ) 15 December 1981, see abstract.	1-21
Y,P	WO, A, 90/13296 (FLOYD) 15 November 1990, see example 1.	1-21
<p>* Special categories of cited documents ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority of an invention which is cited to establish the publication date of an invention or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>¹¹ The document published with the international filing date or priority date shall be cited with a reference to the date of publication or the date of the application which defined the invention</p> <p>¹² Documents of particular interest to the applicant or to the public shall be cited with a reference to the relevant passages</p> <p>¹³ Documents of particular interest to the applicant or to the public shall be cited with a reference to the relevant passages</p> <p>¹⁴ Documents of particular interest to the applicant or to the public shall be cited with a reference to the relevant passages</p> <p>¹⁵ Documents of particular interest to the applicant or to the public shall be cited with a reference to the relevant passages</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this Search Report
10 July 1991		06 SEP 1991
International Searching Authority		Signature of Authority
ISA/US		Sandra Saucier